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Example 4. Construction of an adenovirus mutated in VAI and VAI RNA genes.

To delete both VAI and VAI RNA from the adenovirus genome, restriction enzymes Xba I and BspE I were used applying standard molecular biology techniques. Xba I (sequence TCTAGA) cuts 4 times along the adenovirus type 5 genome and one of those cuts is located 30 bp before the VAI RNA gene (see Figure 2). BspE I (sequence TCCGGA) cuts 9 times along the adenovirus type 5 genome and one of those cuts is located 20 bp before the end of the VAI RNA gene (see Figure 2). Therefore, these specific Xba I and BspE I sites flank the region from bp 10590 to 11011 of Ad5 that contains the VAI and VAI RNA genes. In order to cut only at these sites, a Sal I fragment of Ad5 was subcloned into a small plasmid (pCT) so these Xba I and BspE I sites become unique sites. After cutting with Xba I and BspE I, the deletion ends were filled with klenow polymerase and religated. This procedure resulted in a 418 bp deletion that removes VAI and VAI RNA genes (named "VAdel" deletion). The deletion point is flanked by the klenow-filled XbaI and BspE I sites: TCTAG-CCGGA. The deletion was transferred to the rest of the adenovirus genome in two steps: one homologous recombination and one ligation of an AscI fragment of the Ad5 genome (see Figure 7 for the complete strategy). The plasmid with the complete Ad5 genome except for the 10590 to 11011 deletion is named pAdVAdel. Virus AdVAdel was obtained from this plasmid by releasing the genome with restriction enzyme Pac I and transfecting it in 293T cells. This cell line was used

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because it is known that SV40 T-antigen can complement defects of Adenovirus VA RNAs. After amplification and purification of virus "AdVAdel" the sequence from its genome shows the deletion of both VAI and VAII (Figure 7 lower pannel).

Example 5. An adenovirus with both VAI and VAII RNAs mutated is more selective for tumor cells with an active Ras pathway than an adenovirus with only the VAI RNA mutated.

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To measure the selectivity of the viruses described in the invention, we compared the IC50 (concentration of virus in viral particles per cell that produces 50% of cell lysis) in cells with an inactive (293 cells) or an active Ras pathway (NP9 cells). The adenoviruses tested were Adwt (no deletions), dl331 (VAI deletion) and AdVAdel (VAI and VAII deletions). The methods are the same as described in Example 2. The results are presented in Figure 8. In 293 cells, AdVAdel was more defective than dl331 and Adwt. Compared to Adwt the defectiveness is in the order of 300-fold. Compared to dl331, AdVAdel is 10-fold more defective in cells with non-active Ras. This defectiveness achieved in cells with non-active Ras pathway was also demonstrated at the level of viral protein production. Structural proteins synthesized at a late phase of the virus replication cycle were detected by western blot using a polyclonal antibody. As shown at the upper right panel of Figure 8 the defectiveness compared to Adwt of the VAI and VAII deleted mutant is clearly observed and very superior to the defectiveness of the VAI mutant dl331. In contrast,

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in NP9 pancreatic carcinoma cells with an active Ras pathway AdVAdel is not defective compared to Adwt and dl331 both at the level of cytolytic effects and late protein expression (Figure 8 lower panels).

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5 To demonstrate that the adenovirus with mutated VAI and VAI1 shows Ras-dependent replication we used transient transfections with active (V12) or negative-dominant (N17) forms of Ras as presented in Example 1. The cytolytic effects of AdVAdel virus were compared to Adwt in each
10 transfected cell population (Figure 9). In mock-transfected (GFP-plasmid control) 293 cells, AdVAdel was 320-fold more defective than Adwt. When the Ras-pathway was activated with Ras-V12 this defect was eliminated. Conversely, a further inhibition of the basal Ras-pathway in 293 cells
15 led to an attenuation of AdVAdel of 3500 times compared to Adwt. This level of Ras-dependent replication measured using this transient transfection assay is much higher than the observed for the single VAI mutant dl331 (20 times as shown in Example 1).

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